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**PRIORITY CLAIM PURSUANT TO 35 U.S.C. §119**

Sir:

Pursuant to 37 C.F.R. §1.55(a) and 35 U.S.C. §119, Applicants hereby claim priority to European patent application no. 125790, filed August 13, 1998 in the Israeli Patent Office. A certified copy of the priority application is submitted herewith.

Respectfully submitted,

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CERTIFICATE OF MAILING

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June 28, 2001
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# דיכוי של תהליכים פתוגניים הקשורים לטראומה רקמתית

# INHIBITION OF PATHOGENIC PROCESSES RELATED TO TISSUE TRAUMA

# INHIBITION OF PATHOGENIC PROCESSES RELATED TO TISSUE TRAUMA

## FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to the inhibition of pathogenic processes associated with tissue trauma by modulation, at the molecular level, of the extracellular matrix economy. More particularly, the present invention relates to compositions containing a quinazolinone derivative which are useful for such modulation, especially the quinazolinone Halofuginone.

The synthesis and deposition of collagen are important underlying processes for the mechanisms of a number of different pathophysiological conditions, including the formation of adhesions, hepatic fibrosis and cirrhosis, the formation of keloids and hypertrophic scars, and pulmonary fibrosis. All of these pathophysiological processes are abnormal responses to tissue trauma.

The physiological response to tissue trauma is a complex process involving such factors as cells, extracellular matrix (ECM) components and the cellular microenvironment. Essentially, such a response involves the repair or replacement of damaged tissues. The precise nature of such repair or replacement depends upon the tissues involved, although all such processes involve certain basic principles. The pathophysiological response to the tissue trauma may differ in these tissues as well, but often involves the formation of adhesions or other types of abnormal tissues which do not duplicate the functionality of the original organ tissue, so that

the repair of tissue trauma does not lead to a complete restoration of organ capacity and function.

For example, the repair of damaged skin involves the reconstruction of one or more skin layers. Skin has three layers, keratin, epidermis and dermis. If all three skin layers are damaged or destroyed, new connective tissue, called granulation tissue, must first fill the wound space. This tissue is formed by deposition of ECM components by fibroblasts, which migrate into the wound space [D.R. Knighton and V.D. Fiegel, *Invest. Radiol.*, Vol 26, p. 604-611, 1991]. Although the formation of granulation tissue is clearly an important protective mechanism, the formation of such tissue can also lead to the formation of scars. Production of ECM components, such as collagen, has been particularly linked to scar formation. Since these scars do not replicate the exact functionality and appearance of the original skin tissue, they can lead to both cosmetic and functional problems.

Other types of abnormal skin tissues include keloids and hypertrophic scars. Keloids are benign fibrotic tumors which are believed to arise from the reticular dermis. They are characterized by increased tissue fibrosis and collagen deposition [Friedman, D.W. *et al.*, *J. Surg. Res.*, Vol. 55, p. 214-222, 1993], and are often associated with trauma. They occur most commonly on the upper back, anterior chest, shoulders and ear lobes. Hypertrophic scars are somewhat related to keloids, in that they are also characterized by increased tissue fibrosis and collagen deposition [Friedman, D.W. *et al.*, *J. Surg. Res.*, Vol. 55, p. 214-222, 1993].

Keloids and hypertrophic scars are characterized histologically by a rich vasculature, a high mesenchymal cell density, a thickened epidermis cell layer, and an abundance of collagen fibers. In hypertrophic scars, these fibers are loosely arrayed in a swirl-like pattern within bundles. In keloids, these fibers show even less organization, without any discrete bundles. By contrast, in normal skin these collagen fibers are arranged in distinct, clearly demarcated bundles. Collagen production, as measured by prolyl hydroxylase activity, was found to be elevated in keloids, as compared to normal skin and normally healing wounds [Cohen, K.I. *et al.*, *Surg. Forum*, Vol 22, p. 488, 1971]. Collagen synthesis was also found to be elevated in hypertrophic scars, but not to as great an extent [Rockwell, W.B. *et al.*, *Plastic and Recon. Surg.*, Vol. 84, p. 827-835, 1989]. The ratio of type I collagen to type III collagen was found to be significantly elevated in keloids but not hypertrophic scars, due to a specific increase in $\alpha 1$(I) collagen gene expression, although type III collagen gene expression is also increased [Friedman, D.W. *et al.*, *J. Surg. Res.*, Vol. 55, p. 214-222, 1993; Rockwell, W.B. *et al.*, *Plastic and Recon. Surg.*, Vol. 84, p. 827-835, 1989]. Thus, clearly the deposition of collagen plays an important role in keloid and hypertrophic scar formation.

Other tissues which are damaged by tissue trauma can also undergo such pathophysiological processes, with resultant formation of abnormal tissues. For example, various organ tissues can be damaged by a bacterial infection or by surgical procedures, which can result in the formation of adhesions. Adhesions can cause a number of further complications, such as intestinal obstruction, infertility, pelvic pain, and other related dysfunction. The formation of adhesions between

organs of the abdominal or pelvic cavities is a frequent and undesirable complication of abdomino-pelvic surgery. Surgical trauma to the tissue causes the release of a serosanguinous exudate, which forms a fibrous bridge persisting over the 4-5 days required for remesothelialization [Hill-West, J.L., *et al.*, *Obstet. Gynecol.*, Vol 83, p. 59-64, 1994; Sawhney, A.S., *et al.*, *Macromolecules*, Vol. 26, p. 581-587, 1993]. If the exudate is not absorbed or lysed within this period, it becomes ingrown with fibroblasts. Subsequent collagen production and deposition from these fibroblasts directly causes the formation of permanent scar tissue, which can connect the traumatized tissue to another organ, for example [Mahadevan *et al.*, *Fertil. Steril.*, Vol. 44, p. 489-92, 1985]. Such permanent scar tissue is called an adhesion. Thus, similar to the pathogenic processes associated with the repair of traumatized skin tissue, the formation of adhesions is also associated with excessive and unwanted collagen synthesis.

In addition, both pulmonary and hepatic tissues can also be damaged by these pathophysiological responses to tissue trauma, which can include the formation of fibrotic tissues in the lungs or liver. Pulmonary fibrosis is a chronic and incurable disease in which interstitial connective tissue accumulates in the lungs, reducing lung functionality and efficiency of gas exchange [S. Phan, *New Strategies for Treatment of Pulmonary Fibrosis*, **50**:415-421, 1995]. The fibrotic tissue replaces more complex pulmonary tissue in a pathological process which progressively reduces the surface area for gas exchange in the lungs. Pulmonary fibrosis often follows such therapeutic interventions as bone marrow transplantation, radiotherapy and

chemotherapy [Nagler, A. *et al.*, *Am. J. Respir. Crit. Care Med.*, 154:1082-1086, 1996]. The disease is frequently fatal.

The pathogenesis of pulmonary fibrosis includes the formation of fibrotic tissue in the lungs. The formation of fibrotic tissue is characterized by the deposition of abnormally large amounts of collagen. Similarly, in hepatic fibrosis, connective tissue accumulates in the liver, replacing normal hepatic parenchymal tissue, and reducing liver functionality. The fibrotic tissue replaces more complex normal liver tissue in a pathological process which reduces the amount of liver tissue available for normal functions, such as the removal of toxic substances from the blood, and which progressively disrupts intrahepatic blood flow. The formation of fibrotic tissue in the liver is characterized by the deposition of abnormally large amounts of extracellular matrix components, including at least five types of collagen, in particular collagen types I, III, and IV, as well as other matrix proteins [L. Ala-Kokko, *Biochem. J.*, 244:75-9, 1987]. Thus, again the excessive, abnormal and undesirable synthesis of collagen plays a vital role in the development of pulmonary and hepatic fibrosis, both of which are pathogenic responses to tissue trauma.

Indeed, one common factor in all of these pathogenic responses to tissue trauma is the synthesis of collagen. The synthesis of collagen is also involved in a number of other pathological conditions. For example, clinical conditions and disorders associated with primary or secondary fibrosis, such as systemic sclerosis, graft-versus-host disease (GVHD), pulmonary and hepatic fibrosis

and a large variety of autoimmune disorders, are distinguished by excessive production of connective tissue, which results in the destruction of normal tissue architecture and function. These diseases can best be interpreted in terms of perturbations in cellular functions, a major manifestation of which is excessive collagen synthesis and deposition. The crucial role of collagen in fibrosis and other pathological conditions has prompted attempts to develop drugs that inhibit its accumulation [K.I. Kivirikko, *Annals of Medicine*, Vol. 25, pp. 113-126 (1993)].

Such drugs can act by modulating the synthesis of the procollagen polypeptide chains, or by inhibiting specific post-translational events, which will lead either to reduced formation of extra-cellular collagen fibers or to an accumulation of fibers with altered properties. Unfortunately, only a few inhibitors of collagen synthesis are available, despite the importance of this protein in sustaining tissue integrity and its involvement in various disorders.

For example, cytotoxic drugs have been used in an attempt to slow the proliferation of collagen-producing fibroblasts [J.A. Casas, *et al.*, *Ann. Rhem. Dis.*, **46**: 763, 1987], such as colchicine, which slows collagen secretion into the extracellular matrix [D. Kershenobich, *et al.*, *N. Engl. J. Med.*, **318**:1709, 1988], as well as inhibitors of key collagen metabolism enzymes [K. Karvonen, *et al.*, *J. Biol Chem.*, **265**: 8414, 1990]; C.J. Cunliffe, *et al.*, *J. Med. Chem.*, **35**:2652 ,1992].

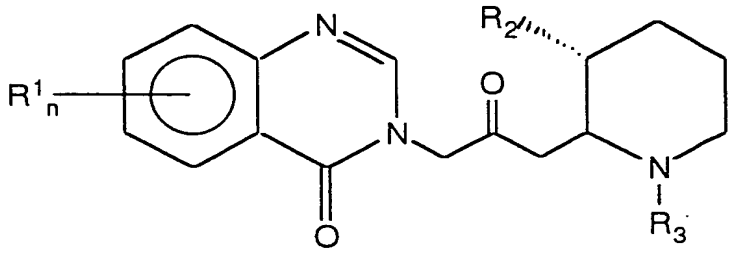
Unfortunately, none of these inhibitors are collagen-type specific. Also, there are serious concerns about the toxic consequences of interfering with

biosynthesis of other vital collagenous molecules, such as Clq in the classical complement pathway, acetylcholine esterase of the neuro-muscular junction endplate, conglutinin and pulmonary surfactant apoprotein.

Other drugs which can inhibit collagen synthesis, such as nifedipine and phenytoin, inhibit synthesis of other proteins as well, thereby non-specifically blocking the collagen biosynthetic pathway [T. Salo, *et al.*, *J. Oral Pathol. Med.*, **19**: 404 ,1990].

Collagen cross-linking inhibitors, such as β-amino-propionitrile, are also non-specific, although they can serve as useful anti-fibrotic agents. Their prolonged use causes lathritic syndrome and interferes with elastogenesis, since elastin, another fibrous connective tissue protein, is also cross-linked. In addition, the collagen cross-linking inhibitory effect is secondary, and collagen overproduction has to precede its degradation by collagenase. Thus, a type-specific inhibitor of the synthesis of collagen itself is clearly required as an anti-fibrotic agent.

Such a type-specific collagen synthesis inhibitor is disclosed in U.S. Patent No. 5,449,678 for the treatment of a fibrotic condition. This specific inhibitor is a composition with a pharmaceutically effective amount of a pharmaceutically active compound of a formula:



wherein:

$R_1$ is a member of the group consisting of hydrogen, halogen, nitro, benzo, lower alkyl, phenyl and lower alkoxy;

$R_2$ is a member of the group consisting of hydroxy, acetoxy and lower alkoxy, and

$R_3$ is a member of the group consisting of hydrogen and lower alkenoxy-carbonyl; as well as pharmaceutically acceptable salts thereof. Of this group of compounds, Halofuginone has been found to be particularly effective for such treatment.

U.S. Patent No. 5,449,678 discloses that these compounds are effective in the treatment of fibrotic conditions such as scleroderma and GVHD. WO Application No. 96/06616 further discloses that these compounds are effective in treating restenosis. The two former conditions are associated with excessive collagen deposition, which can be inhibited by Halofuginone. Restenosis is characterized by smooth muscle cell proliferation and extracellular matrix accumulation within the lumen of affected blood vessels in response to a vascular injury [Choi *et al.*, *Arch. Surg.*, **130**:257-261, 1995]. One hallmark of such smooth muscle cell proliferation is a phenotypic alteration, from the normal contractile phenotype to a synthetic one. Type I collagen has been shown to support such a phenotypic alteration, which can be blocked by Halofuginone [Choi *et al.*, *Arch. Surg.*, **130**: 257-261, 1995; U.S. Patent No. 5,449,678].

However, the *in vitro* action of Halofuginone does not always predict its *in vivo* effects. For example, Halofuginone inhibits the synthesis of collagen type I in bone chrondrocytes *in vitro*, as demonstrated in U.S. Patent No. 5,449,678. However, chickens treated with Halofuginone were not reported to have an increased rate of bone breakage, indicating that the effect is not seen *in vivo*. Thus, the exact behavior of Halofuginone *in vivo* cannot always be accurately predicted from *in vitro* studies.

Indeed, the initial discovery of the ability of Halofuginone to successfully treat several different disease states was completely serendipitous. Halofuginone has been shown to be effective for these disease states by trial-and-error, since the precise underlying mechanism of action of Halofuginone was not known. Such a lack of knowledge, coupled with the inability to completely predict the *in vivo* behavior of Halofuginone from its *in vitro* effects, has limited the development of new agents for these pathophysiological conditions.

The elucidation of the underlying mechanism of the action(s) of Halofuginone would enable new and potentially even more effective treatments to be developed. Furthermore, such treatments could be designed to precisely pinpoint the molecular targets of Halofuginone and other quinazolinone derivatives, thereby potentially reducing the unwanted side effects of treatment. Such treatments could also regulate the overall extracellular matrix economy, and thus lead to the amelioration of many different pathological conditions associated with disturbances in this economy.

There is thus a widely recognized unmet medical need for specific effectors capable of regulating the extracellular matrix economy, whose mechanism of action includes targeted intervention at the transcriptional or other molecular level, such that a specific effect on the pathological responses to tissue trauma is determined by a precise intervention at a particular molecular target.

## SUMMARY OF THE INVENTION

Unexpectedly, it has been found, as described in the examples below, that the underlying mechanism of action of Halofuginone in the inhibition of all of these pathogenic responses to tissue trauma involves the regulation of the extracellular matrix economy at the molecular level. One important molecular target of Halofuginone is the *cKrox* transcription factor for the expression of the collagen α1(I) gene. *cKrox* is a novel zinc finger-containing transcription factor which binds to the α1(I) and α2(I) collagen gene promoters, and was shown to repress transcription of the α1(I) procollagen promoter (Widom R L; Culic I; Lee J Y; Korn J H; *GENE*, (1997 Oct 1) **198**:407-20). As described in further detail in the examples below, Halofuginone, in turn, was shown to potentiate the effect of *cKrox* and thus to potentiate inhibition of collagen synthesis.

Other potential molecular targets for Halofuginone include the inhibition of collagenase type IV activity and the inhibition of H19 gene expression, as well as the overall regulation of ECM (extracellular matrix) deposition and remodelling, and the inhibition of neo-angiogenesis. These targets, however, are likely to either be secondary targets for the mechanism of action of Halofuginone, or to

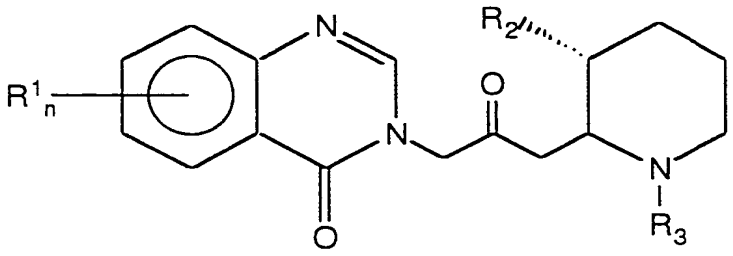
only be indirectly inhibited by Halofuginone. Indeed, these inhibitory effects may in turn be related to the potentiation of the c*Krox* transcriptional factor.

In any case, all of these mechanisms are related to the regulation of the "extracellular matrix economy". Such regulation is not merely inhibition of all processes related to the extracellular matrix and collagen deposition, since Halofuginone was previously shown by the inventors to inhibit excessive collagen deposition associated with keloids and other abnormal scar formation, yet Halofuginone did not decrease wound strength, as shown U.S. Application No. 08/797,701, filed on February 11, 1997, incorporated by reference as if fully set forth herein. Instead the term "extracellular matrix economy" is intended to indicate the constellation of processes related to the synthesis, deposition and maintenance of the extracellular matrix and related tissue structures. The proper regulation of the extracellular matrix economy leads to the inhibition of the pathological response to tissue trauma, such that all of the potential targets for the mechanism of action of Halofuginone and other effectors are able to prevent such pathological responses substantially without inhibiting or altering other desirable physiological activity.

According to one embodiment of the present invention, there is provided a composition for regulating at least one function of the extracellular matrix economy, comprising a pharmaceutically effective amount of an effector in combination with a pharmaceutically acceptable carrier. Preferably, the at least one function of the extracellular matrix economy includes promotion of an activity of *cKrox* transcription factor. More preferably, the activity of the

*cKrox* transcription factor is promoted by increasing expression of *cKrox* transcription factor gene.

According to a preferred embodiment of the present invention, the effector is a quinazolinone derivative. Preferably, the quinazolinone derivative is a member of a group having a formula:




wherein:

$R_1$ is a member of the group consisting of hydrogen, halogen, nitro, benzo, lower alkyl, phenyl, and lower alkoxy;

$R_2$ is a member of the group consisting of hydroxy, acetoxy, and lower alkoxy, and

$R_3$ is a member of the group consisting of hydrogen and lower alkenoxy;

and pharmaceutically acceptable salts thereof. More preferably, the effector is Halofuginone and pharmaceutically acceptable salts thereof.

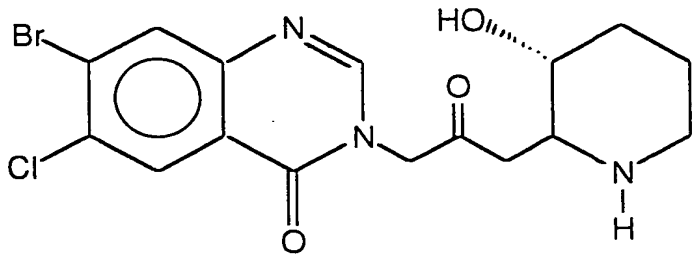
According to another embodiment of the present invention, there is provided a composition for inhibition of at least one pathological process associated with tissue trauma or a mechanistically related process thereof, comprising a pharmaceutically effective amount of an effector in combination with a pharmaceutically acceptable carrier, wherein the effector regulates at least one function of the extracellular matrix economy in order to inhibit the at

least one pathological process associated with tissue trauma or a mechanistically related process thereof.

According to preferred embodiments of the present invention, the at least one pathological process is selected from the group consisting of cancers, fibrotic conditions including but not limited to hepatic fibrosis and cirrhosis, pulmonary fibrosis, cardiac fibrosis, neo-angiogenesis, formation of adhesions, psoriasis, keloids, hypertrophic scars, and any other such pathological condition which can be ameliorated, reduced or otherwise treated by an effector capable of regulating the extracellular matrix economy.

According to yet another embodiment of the present invention, there is provided a composition for inhibition of cardiac fibrosis, comprising a pharmaceutically effective amount of an effector in combination with a pharmaceutically acceptable carrier, wherein the effector is capable of inhibiting cardiac fibrosis.

Hereinafter, the term "Halofuginone" is defined as a compound having a formula:




and pharmaceutically acceptable salts thereof. The composition preferably includes a pharmaceutically acceptable carrier for the compound.

Preferably, all of the compounds referred to hereinabove can be either the compound itself as described by the formula, and/or pharmaceutically acceptable salts thereof.

## BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 illustrates certain exemplary aspects of the extracellular matrix economy;

FIG. 2 illustrates the dose-dependent inhibition of type IV collagenase activity in T50 bladder carcinoma cell cultures in the presence of Halofuginone;

FIG. 3 illustrates the inhibition of the expression of the H19 gene in the RT112 and 5376 bladder carcinoma cell lines by Halofuginone;

FIG. 4 shows a Northern blot with the effect of Halofuginone on Integrin $\alpha_v$ chain expression;

FIG. 5 shows the effect of Halofuginone on β subunit expression as determined by RT-PCR; and

FIG. 6 shows the ability of Halofuginone to inhibit cardiac fibrosis.

## BRIEF DESCRIPTION OF THE INVENTION

Unexpectedly, it has been found, as described in the examples below, that the underlying mechanism of action of Halofuginone in the inhibition of all

of these pathogenic responses to tissue trauma involves the regulation of the extracellular matrix economy at the molecular level. One important molecular target of Halofuginone is the *cKrox* transcription factor for the expression of the collagen α1(I) gene. *cKrox* is a novel zinc finger-containing transcription factor which binds to the α1(I) and α2(I) collagen gene promoters, and was shown to repress transcription of the α1(I) procollagen promoter (Widom R L; Culic I; Lee J Y; Korn J H; *GENE*, (1997 Oct 1) **198**:407-20). As shown in the examples below, Halofuginone, in turn, was shown to potentiate the effect of *cKrox* and thus to potentiate inhibition of collagen synthesis.

*c-Krox* was also found to be present at much higher levels in skin than bone, which may explain why Halofuginone was able to decrease excessive collagen deposition in skin while not increasing the fragility of bone (Galera P; Musso M; Ducy P; Karsenty G; *PNAS*, (1994 Sep 27) **91**: 9372-6).

Other potential molecular targets for Halofuginone and other effectors include the inhibition of collagenase type IV activity and the inhibition of H19 gene expression, as well as the overall regulation of ECM (extracellular matrix) deposition and remodelling, and the inhibition of neo-angiogenesis. These targets, however, are likely to either be secondary targets for the mechanism of action of Halofuginone, or to only be indirectly inhibited by Halofuginone. Indeed, these inhibitory effects may in turn be related to the potentiation of the *cKrox* transcriptional factor.

In any case, all of these mechanisms are related to the regulation of the "extracellular matrix economy". Such regulation is not merely inhibition of all

processes related to the extracellular matrix and collagen deposition, since Halofuginone was previously shown by the inventors to inhibit excessive collagen deposition associated with keloids and other abnormal scar formation, yet Halofuginone did not decrease wound strength, as shown in U.S. Application No. 08/797,701, filed on February 11, 1997, incorporated by reference as if fully set forth herein.

The proper regulation of the extracellular matrix economy leads to the inhibition of the pathological response to tissue trauma, such that all of the potential targets for the mechanism of action of Halofuginone and other effectors are able to prevent such pathological responses substantially without inhibiting or altering other desirable physiological activity. Indeed, the term "effector" is used herein to refer to substantially any compound or combination thereof which is capable of regulating the extracellular matrix economy, thereby specifically inhibiting pathological processes related to tissue trauma and mechanistically related processes. The term "mechanistically related processes" includes those pathological conditions which share at least one underlying mechanism with abnormal responses to tissue trauma. For example, the metastasis of malignant cells is an example of a mechanistically related process, because such metastasis depends upon neo-angiogenesis, which in turn depends upon the deposition of extracellular matrix components including collagen, as shown in U.S. Application No. 08/797,703, filed on February 11, 1997, and PCT Application No. IL98/00070, filed on February 11, 1998, both of which are incorporated by reference as if fully set forth herein. Similarly,

abnormal responses to tissue trauma such as adhesion formation also depend upon collagen deposition. Thus, all of these pathological processes can be said to be mechanistically related.

Other mechanisms of the "extracellular matrix economy" include, but are not limited to, the inhibition of angiogenesis, the prevention of ECM deposition, the inhibition of collagenase type IV activity, the inhibition of integrin expression, the induction of apoptosis and the inhibition of H19 gene expression. The overall structure of the extracellular matrix economy, and the effect of Halofuginone in relation to this economy, is shown in Figure 1. As shown, the specific effects of Halofuginone on the extracellular matrix economy include the inhibition or amelioration of conditions associated with tumors, fibrosis, scleroderma and GVHD, restenosis and skin injury. By mediating certain effects or factors associated with the extracellular matrix economy, Halofuginone and other effectors are able to inhibit collagen type I synthesis, hence ameliorating pathological conditions associated with tissue trauma while permitting normal, desirable physiological processes to continue.

With regard to specific aspects of these mechanisms, angiogenesis and ECM deposition have been previously described. Collagenase type IV is a pivotal metalloprotease enzyme involved in metastasis and cell invasion. Apoptosis is programmed cell death which, as noted previously, is blocked in malignant cells, which are therefore also described as "immortal". The H19 gene is a tumor-marker gene associated with the early stages of bladder carcinoma. More specifically, the H19 gene is a developmentally regulated gene whose expression peaks during fetal

development when tissue differentiation is occurring. Chromosomal abnormalities within the region containing H19 are associated with early stages of malignancies such as Wilms' tumor, adrenocortical carcinoma, hepatoblastoma, rhabdomyosarcoma, lung tumors, trophoblastic tumors and bladder carcinoma [B. Tycko, *Am. J. Path.*, Vol. 144, p. 431-439, 1994; de Groot, N. *et al.*, *Trophoblast Res.*, Vol. 8, p. 2285-2302, 1994; Rachmilewitz, J. *et al.*, *Oncogene*, Vol. 11, p. 863-870, 1995].

The importance of extracellular matrix economy is that the mechanism of action of Halofuginone is able to promote many desirable activities, such as the cytostatic inhibition of malignancies, the reduction or prevention of fibrosis and adhesions, and other pathological responses to tissue trauma, or mechanistically related activities thereof, substantially without causing undesirable side effects such as decreasing the strength of healed wounds or altering the remodelling of the structure of bone.

## DESCRIPTION OF PREFERRED EMBODIMENTS

Unexpectedly, it has been found, as described in the examples below, that the underlying mechanism of action of Halofuginone in the inhibition of all of these pathogenic responses to tissue trauma involves the regulation of the extracellular matrix economy at the molecular level, specifically by enhancing the activities of the *cKrox* transcription factor, which in turn represses transcription of the $\alpha$1(I) procollagen promoter.

Furthermore, Halofuginone is also involved in other aspects of the extracellular matrix economy, including the inhibition of collagenase type IV activity and the inhibition of H19 gene expression, as well as the overall regulation of ECM (extracellular matrix) deposition and remodelling, and the inhibition of neo-angiogenesis. Other mechanisms of the "extracellular matrix economy" include, but are not limited to, the inhibition of angiogenesis, the prevention of ECM deposition, the inhibition of collagenase type IV activity, the inhibition of integrin expression, the induction of apoptosis and the inhibition of H19 gene expression. Such specific regulation of the extracellular matrix economy has never been demonstrated before, particularly *in vivo*.

While the invention will now be described in connection with certain preferred embodiments in the following figures and examples so that aspects thereof may be more fully understood and appreciated, the invention is not intended to be limited to these particular embodiments. On the contrary, all alternatives, modifications and equivalents are included as within the scope of the invention as defined by the appended claims. Thus, the following figures and examples which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention.

The present invention may be more readily understood with reference to the following illustrative examples and figures. It should be noted that although reference is made exclusively to Halofuginone, it is believed that the other quinazolinone derivatives described and claimed in U.S. Patent 3,320,124, the teachings of which are incorporated herein by reference, have similar properties.

## Example 1
## Promotion of *cKrox* Activity and Inhibition of Collagen Type I Gene Expression by Halofuginone

As noted previously, one of the most important targets for the action of Halofuginone and other related quinazolinones and effectors is the promotion of *cKrox* activity and the concomitant inhibition of collagen type I gene expression. These two activities were demonstrated with Halofuginone as follows.

First, the ability of Halofuginone to inhibit collagen type I gene expression was demonstrated as follows. Myometrial and leiomyosarcoma cells were taken from the same patient and were plated into 10 cm plates in DMEM supplemented with 10% FCS. When the cells reached 80% confluence, the medium was replaced by serum free DMEM plus 0.1% BSA for 48 hours, washed and exposed to increasing concentrations of Halofuginone in the same medium for about 48 hours at about 37 °C. The cells were then harvested and subjected to RNA extraction and Northern blot analysis for collagen type I gene

expression. Halofuginone inhibited collagen type I gene expression (products at 5.4 and 4.8 kb) in a dose-dependent manner.

Next, human skin fibroblasts were taken from a subject and were maintained in primary culture. Control cells were treated with vehicle while Halofuginone-treated cells were treated with Halofuginone as previously described. The cells were then harvested and subjected to RNA extraction and Northern blot analysis for collagen type I gene expression and for *cKrox* gene expression. Halofuginone promoted *cKrox* gene expression while simultaneously inhibiting collagen type I gene expression. Thus, one important molecular target for the mechanism of action of Halofuginone is clearly the enhancement of *cKrox* gene expression and hence *cKrox* activity, which in turn leads to the inhibition of collagen type I gene expression.

Such targeting of the action of Halofuginone is novel, and has not been taught nor suggested by the background art. However, clearly the elucidation of this mechanism is important for the development and design of new treatments for the pathological processes associated with tissue trauma. Furthermore, such results provide a clear mechanistic explanation for the ability of Halofuginone to inhibit pathological collagen synthesis, while enabling normal physiological processes associated with collagen to proceed without unwanted side effects. In particular, molecules and chemical compositions which also are able to inhibit pathological collagen synthesis, while enabling normal physiological processes associated with collagen to proceed without unwanted side effects, are now possible by targeting the

potentiation of *cKrox* gene expression and/or activity for therapeutic intervention.

## Example 2
## Inhibition of Type IV Collagenase Activity by Halofuginone *in vitro*

Another important feature of the regulation of the extracellular matrix economy is the inhibition of type IV collagenase activity by Halofuginone.

Tumor cells secrete enzymes which digest the ECM, enabling the cells to burrow through neighboring tissue and to invade other tissues. Numerous studies have linked matrix metalloproteases (MMP), especially type IV collagenase, to the process of tumor invasion and metastasis. Type IV collagenase appears as two 72 and 92 kDa proteins encoded by a unique mRNA.

As demonstrated in Figure 2, a profound inhibition of the activity of MMP2 (72 kDa type IV collagenase) in T50 bladder carcinoma cell cultures was exerted in the presence of 25 ng/ml Halofuginone, while an almost complete inhibition was obtained at 100 ng/ml Halofuginone. Sub-confluent cell cultures were incubated for 6 - 24 h in serum-free DMEM. The collagenolytic activity was determined on a gelatin impregnated (1 mg/ml. Difco, Detroit, MI) SDS-PAGE 8% gel. Briefly, culture media samples were separated on the substrate impregnated gels under non reducing conditions, followed by 30 min incubation in 2.5% Triton X-100 (BDH, England). The gels were then incubated for 16 h at 37°C in 50 mM Tris, 0.2 M NaCl, 5 mM

$CaCl_2$, 0.02% Brij 35 (weight/volume) at pH 7.5. At the end of incubation period, the gels were stained with 0.5% Coomassie G250 (Bio-Rad Richmond CA) in methanol/acetic acid/$H_2O$ (30:10:60). The intensity of the various bands was determined on a computerized densitometer (Molecular Dynamics type 300A).

Halofuginone was also found to inhibit cell invasion through matrigel ECM, using the Boyden chamber invasion assay (data not shown). Such inhibition supports the inclusion of type IV collagenase inhibition as part of the extracellular matrix economy mechanism, in which Halofuginone inhibits undesirable pathological processes such as tumor growth, progression and metastasis, as described previously.

## Example 3
## Inhibition of Tumor-Marker Gene Expression by Halofuginone *in vitro*

Yet another aspect of the extracellular matrix economy is the regulation of tumor-marker gene expression, and in particular of the inhibition of the expression of the H19 gene.

The H19 gene is a developmentally regulated gene whose expression peaks during fetal development when tissue differentiation is occurring. The H19 gene is parentally imprinted, expressed only by the maternal allele. H19 is also a tumor-marker gene, associated with early stages of malignancies such as Wilms' tumor, adrenocortical carcinoma, hepatoblastoma,

rhabdomyosarcoma, lung tumors, trophoblastic tumors and bladder carcinoma. The experimental method was as follows.

RT112 and 5376 human bladder carcinoma cell lines were cultured in the absence and presence of Halofuginone (130 ng/ml, added 24 h or 72 h after seeding), and the expression of the H19 gene was evaluated by Northern blot analysis (Nagler, A. *et al.*, *Arterioscler. Thromb. Vasc. Biol.*, Vol. 17, p. 194-202, 1997). Exposure to Halofuginone resulted in a substantial reduction in the expression of the H19 gene in the RT112 and 5376 bladder carcinoma cell lines which were tested, as shown in Figure 3. Such inhibition also supports the inclusion of the inhibition of H19 gene expression as part of the extracellular matrix economy mechanism, as described previously.

## Example 4
## Inhibition of Integrin Expression

Another important aspect of the effect of Halofuginone and related quinazolinones on the extracellular matrix economy involves the ability of these compounds to inhibit integrin expression, as exemplified by the effect of the illustrative compound Halofuginone.

Integrins have been shown to function *in vivo* in vasculogenesis and angiogenesis. Injection of neutralizing antibody against the ß1 subunit blocked the formation of an aortic lumen in quail embryos (Drake, C.J. *et al.*, *in vivo. Dev. Dyn.* Vol. 193, p. 83-91, 1992). Cheresh and colleagues have provided evidence that $\alpha_V\beta_3$ is required for blood vessel growth (Brooks, P.C. *et al.*,

*Science* Vo. 264, p. 569-571, 1994). An antibody (LM609) against the $\alpha_v\beta_3$ integrin complex inhibited normal vessel growth and also FGF-2 stimulated or tumor-induced angiogenesis in the CAM assay, but did not disrupt preexisting vessels. The mechanism by which anti-$\alpha_v\beta_3$ mAb disrupts angiogenesis appears to involve apoptosis. A single intravascular injection of a cyclic RGD peptide antagonist of $\alpha_v\beta_3$ integrin or of the LM609 monoclonal antibody leads to the rapid regression of human tumors transplanted into the CAM. Tumor cells that fail to express the $\alpha_v$ gene and hence the $\alpha_v\beta_3$ integrin, lose their adhesion capability and exhibit a significantly reduced tumorigenicity upon transplantation into athymic nude mice. Stable transfection of the $\alpha_v$ cDNA to these cells resulted in the full restoration of their tumorigenic potential (Felding-Habermann, B. *et al*, *J. Clin. Invest.*, Vol. 89, p. 2018-2022, 1992). Furthermore, Halofuginone has been found to inhibit angiogenesis and tumor growth.

Therefore, the effect of Halofuginone was investigated on the expression of $\alpha_v$, $\beta_3$ and $\beta_5$ integrin subunits in the highly aggressive MDA 435 human breast carcinoma cell line. Cells were cultured in the absence (Figure 4, lanes A & B) or presence of increasing concentrations (10-400 ng/ml) of Halofuginone for 24 h (lane C: 400 ng/ml), 48 h (lanes D-G: 10, 50, 200, and 400 ng/ml, respectively) or 72 h (Figure 4, lane H: 400 ng/ml). Total RNA was extracted, subjected to 1.1% formaldehyde-agarose gel electrophoresis, transferred to a nylon membrane and hybridized with $^{32}$P-labeled PCR probe corresponding to

$\alpha_V$. As demonstrated in Figure 4, exposure of the breast carcinoma cells for 48 h to 10 and 50 ng/ml Halofuginone resulted in up regulation of the $\alpha_V$ mRNA (Figure 4, lanes D & E). This effect was minimal at higher concentrations (200-400 ng/ml) of Halofuginone (Figure 4, lanes F-H). Next, RT-PCR was used to analyze the effect of Halofuginone on expression of the ß3 and ß5 integrin chains by the MDA 435 breast carcinoma cells. As shown in Figure 5, Halofuginone inhibited the expression of the $ß_3$ mRNA in a dose-dependent manner, yielding an almost complete inhibition at 200 ng/ml (Figure 5, lane 1: control; lanes 2-5: 24 h exposure to 10, 50, 200 and 400 ng/ml Halofuginone, respectively). In contrast, there was no effect on expression of the ß5 mRNA. As the $\alpha_V$ß3 integrin complex plays an important role in tumor angiogenesis, the anti-angiogenic effect of Halofuginone may be mediated in part by its inhibition of the ß3 gene expression.

Thus, the inhibition of the expression of integrin genes is clearly another aspect of the regulation of the extracellular matrix economy by Halofuginone.

## Example 5
## Inhibition of Cardiac Fibrosis

As noted previously, the ability of certain molecules to regulate the extracellular matrix economy by inhibiting abnormal responses to tissue trauma and other mechanistically related pathological processes, while maintaining normal physiological processes, has been exemplified by the effect of the

illustrative compound Halofuginone. However, none of these results taught or suggested the ability of Halofuginone to inhibit fibrosis associated with cardiac disease. Such a result is unexpected because cardiac tissue is composed of highly differentiated cells which must maintain a high overall level of organization in order to function effectively.

Furthermore, myocardial tissue must contract as a single unit in response to an electrical signal, which is not a property associated with other previously studied tissues for the treatment of fibrosis with Halofuginone. This property is specific to cardiac tissues, and increases the damaging effect of fibrosis, since fibrotic tissue cannot contract in this manner. Second, damaged myocardial tissue will contract improperly: instead of starting the contraction of the heart at one focal point, followed by the sweep of the potential throughout the heart tissue, arrhythmias can develop in damaged tissues in which many such focal points arise, causing improper contractions and eventually death. Third, the tissue of the heart must function as a single unit. Other tissues, such as lung and liver, are composed of different tissue types and structures which can more or less function independently. However, the entire heart must function as a single unit. Thus, the preservation or restoration of proper myocardial function by Halofuginone, either before or after the fibrotic process has begun, cannot be predicted or taught from the prior art.

The experimental method was performed as follows. Rats were divided into four different groups. The first group of rats, the angiotensin II treated rats, received angiotensin II (AII), a known promoter of cardiac fibrosis. The second group of rats, the treatment group, received both angiotensin II and Halofuginone

(AII + Hal). The third group of rats, the Halofuginone control group, received only Halofuginone (Hal). The last group of rats, the overall control (Con), did not receive Halofuginone or angiotensin II. After the treatment period was ended, the rats were sacrificed and the hearts removed. The collagen volume fraction of each heart was then measured.

As shown in Figure 6, the collagen volume fraction (y-axis) was clearly increased several fold in the angiotensin II treatment group (AII). However, the collagen volume fraction was substantially unchanged in comparison to the control group (Con) in cardiac tissue taken from the treatment group (AII + Hal) or from the rats receiving Halofuginone alone (Hal). Thus, clearly Halofuginone was able to ameliorate cardiac fibrosis while maintaining normal physiological processes in the cardiac tissue.

## Example 6
## Effectors for Regulating the Extracellular Matrix Economy

As clearly demonstrated above, the extracellular matrix economy can be regulated by Halofuginone and other effectors at a number of intervention points. These intervention points enable pathological processes associated with tissue trauma, and other mechanistically related processes, to be selectively inhibited while maintaining substantially normal activity of physiologically desirable processes. As noted previously, the term "mechanistically related processes" refers to those pathological conditions which share one or more underlying mechanisms with the abnormal responses to tissue trauma. An

example of such a mechanistically related process is the growth and metastasis of malignant cancer cells.

The underlying mechanism of action of Halofuginone in the inhibition of all of these pathological processes involves the inhibition of collagen type I synthesis at the molecular level, in particular by promoting *cKrox* activity and hence inhibiting the expression of collagen α1(I) gene expression. The term "promotion of *cKrox* activity" includes, but is not limited to, the upregulation of *cKrox* gene expression and the enhancement of the activity of the *cKrox* protein itself.

Other potential molecular targets for Halofuginone include the inhibition of collagenase type IV activity and the inhibition of H19 gene expression, as well as the overall regulation of ECM (extracellular matrix) deposition and remodelling, and the inhibition of neo-angiogenesis. Other mechanisms of the "extracellular matrix economy" include, but are not limited to, the induction of apoptosis and the inhibition of H19 gene expression. Clearly, the elucidation of these mechanisms and of the overall selective regulation of the extracellular matrix economy demonstrates novel and non-obvious methods for therapeutic intervention which have not been taught nor suggested by the background art.

Therefore, the present invention is also contemplated to include methods and compositions for the regulation of the extracellular matrix economy, and in particular for the promotion of *cKrox* activity. These methods and compositions include the administration of an effector to a subject. The term "effector" as used herein refers to substantially any chemical compound or combination thereof which

is able to regulate the extracellular matrix economy as described above. Preferably, the effector is able to promote *cKrox* activity. More preferably, such promotion is caused by enhancing the expression of the *cKrox* gene.

In preferred embodiments of compositions of the present invention, these compositions include a quinazolinone derivative as the effector. In further preferred embodiments of the present invention, these compositions include Halofuginone as the effector.

The effector of the present invention can be administered to a subject in a number of ways, which are well known in the art. Hereinafter, the term "subject" refers to the human or lower animal to whom the effector was administered. For example, administration may be done topically (including ophtalmically, vaginally, rectally, intranasally), orally, or parenterally, for example by intravenous drip or intraperitoneal, subcutaneous, or intramuscular injection.

Formulations for topical administration may include but are not limited to lotions, ointments, gels, creams, suppositories, drops, liquids, sprays and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, sachets, capsules or tablets. Thickeners, diluents, flavorings, dispersing aids, emulsifiers or binders may be desirable.

Formulations for parenteral administration may include but are not limited to sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Dosing is dependent on the severity of the symptoms and on the responsiveness of the subject to the effector. Persons of ordinary skill in the art can easily determine optimum dosages, dosing methodologies and repetition rates.

The following example is an illustration only of a method of regulating the extracellular economy with an effector such as Halofuginone in order to treat a pathological condition associated with tissue trauma or a mechanistically related condition, and is not intended to be limiting.

The method includes the step of administering the effector, in a pharmaceutically acceptable carrier as described above, to a subject to be treated. The effector is administered according to an effective dosing methodology, preferably until a predefined endpoint is reached, such as a reduction or amelioration of the pathological condition in the subject.

Examples of conditions for which such a treatment would be effective include, but are not limited to, various types of cancers, fibrotic conditions including but not limited to hepatic fibrosis and cirrhosis, pulmonary fibrosis, cardiac fibrosis, neo-angiogenesis, formation of adhesions, psoriasis, keloids, hypertrophic scars, and any other such pathological condition which can be ameliorated, reduced or otherwise treated by an effector capable of regulating the extracellular matrix economy.

While the invention has been described with respect to a limited number of embodiments, it will be appreciated that many variations, modifications and other applications of the invention may be made.

**WHAT IS CLAIMED IS:**

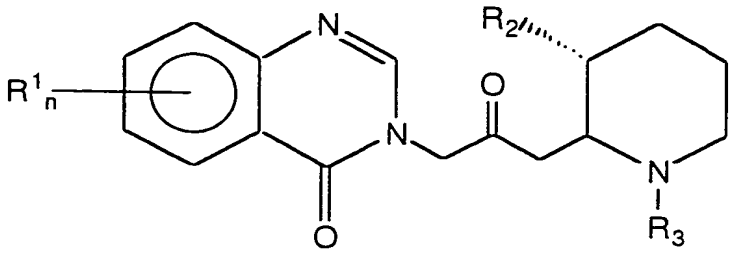
1. A composition for regulating at least one function of the extracellular matrix economy, comprising a pharmaceutically effective amount of an effector in combination with a pharmaceutically acceptable carrier.

2. The composition of claim 1, wherein the at least one function of the extracellular matrix economy includes promotion of an activity of *cKrox* transcription factor.

3. The composition of claim 2, wherein said activity of said *cKrox* transcription factor is promoted by increasing expression of *cKrox* transcription factor gene.

4. The composition of any of claims 1 to 3, wherein said effector is a quinazolinone derivative.

5. The composition of claim 4, wherein said quinazolinone derivative is a member of a group having a formula:



wherein:

$R_1$ is a member of the group consisting of hydrogen, halogen, nitro, benzo, lower alkyl, phenyl, and lower alkoxy;

$R_2$ is a member of the group consisting of hydroxy, acetoxy, and lower alkoxy, and

$R_3$ is a member of the group consisting of hydrogen and lower alkenoxy;

and pharmaceutically acceptable salts thereof.

6. The composition of claim 5, wherein said compound is Halofuginone and pharmaceutically acceptable salts thereof.

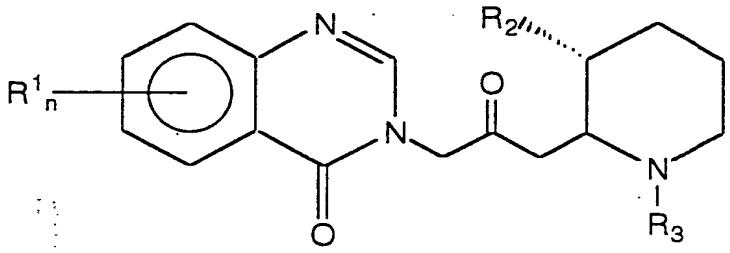
7. A composition for inhibition of at least one pathological process associated with tissue trauma or a mechanistically related process thereof, comprising a pharmaceutically effective amount of an effector in combination with a pharmaceutically acceptable carrier, wherein said effector regulates at least one function of the extracellular matrix economy in order to inhibit the at least one pathological process associated with tissue trauma or a mechanistically related process thereof.

8. The composition of claim 7, wherein said effector promotes an activity of *cKrox* transcription factor.

9. The composition of claim 8, wherein said effector promotes said activity of said *cKrox* transcription factor by increasing expression of *cKrox* transcription factor gene.

10. The composition of any of claims 7 to 9, wherein said effector is a quinazolinone derivative.

11. The composition of claim 10, wherein said quinazolinone derivative is a member of a group having a formula:




wherein:

$R_1$ is a member of the group consisting of hydrogen, halogen, nitro, benzo, lower alkyl, phenyl, and lower alkoxy;

$R_2$ is a member of the group consisting of hydroxy, acetoxy, and lower alkoxy, and

$R_3$ is a member of the group consisting of hydrogen and lower alkenoxy;

and pharmaceutically acceptable salts thereof.

12. The composition of claim 11, wherein said effector is Halofuginone and pharmaceutically acceptable salts thereof.

13. The composition of any of claims 7 to 12, wherein the at least one pathological process is selected from the group consisting of cancers, fibrotic conditions including but not limited to hepatic fibrosis and cirrhosis, pulmonary fibrosis, cardiac fibrosis, neo-angiogenesis, formation of adhesions, psoriasis, keloids, hypertrophic scars, and any other such pathological condition which can be ameliorated, reduced or otherwise treated by an effector capable of regulating the extracellular matrix economy.

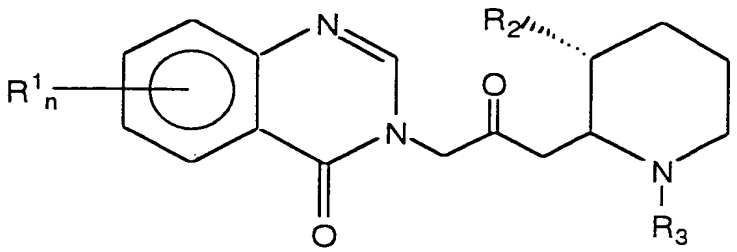
14. A composition for inhibition of cardiac fibrosis, comprising a pharmaceutically effective amount of an effector in combination with a pharmaceutically acceptable carrier, wherein said effector is capable of inhibiting cardiac fibrosis.

15. The composition of claim 14, wherein said effector promotes an activity of *cKrox* transcription factor.

16. The composition of claim 15, wherein said effector promotes said activity of said *cKrox* transcription factor by increasing expression of *cKrox* transcription factor gene.

17. The composition of any of claims 14 to 16, wherein said effector is a quinazolinone derivative.

18. The composition of claim 17, wherein said quinazolinone derivative is a member of a group having a formula:




wherein:

$R_1$ is a member of the group consisting of hydrogen, halogen, nitro, benzo, lower alkyl, phenyl, and lower alkoxy;

$R_2$ is a member of the group consisting of hydroxy, acetoxy, and lower alkoxy, and

$R_3$ is a member of the group consisting of hydrogen and lower alkenoxy;

and pharmaceutically acceptable salts thereof.

19. The composition of claim 18, wherein said effector is Halofuginone and pharmaceutically acceptable salts thereof.

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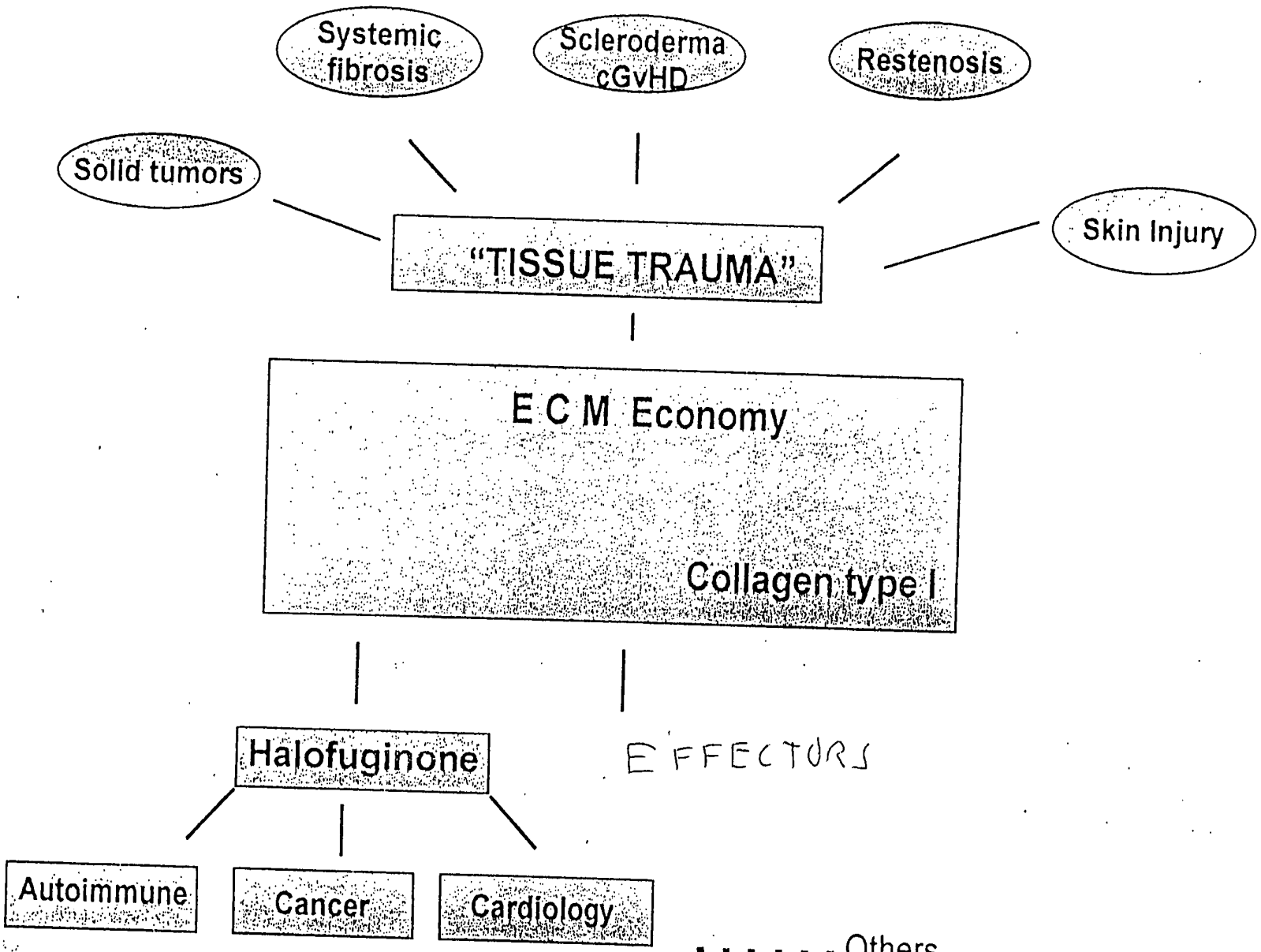
Systemic fibrosis
Scleroderma cGvHD
Restenosis
Solid tumors
"TISSUE TRAUMA"
Skin Injury
E C M Economy
Collagen type I
Halofuginone
EFFECTORS
Autoimmune
Cancer
Cardiology
. . . . . Others

Figure 1

Inhibition of collagenase (MMP2) activity in T50 bladder carcinoma cells by halofuginone.

Halofuginone conc. (ng/ml)
in T50 cells growth medium

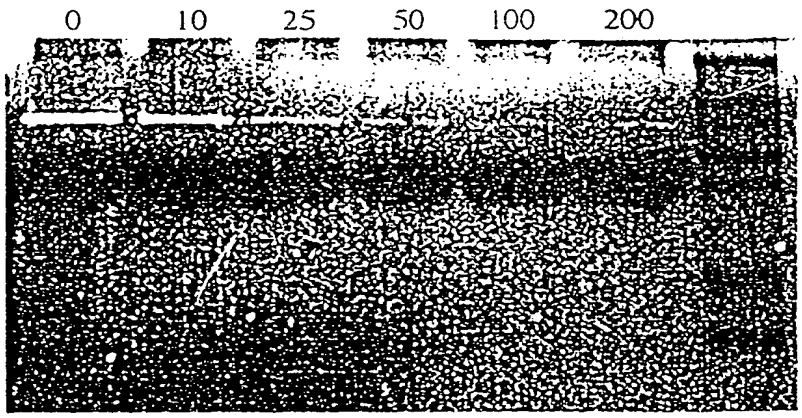


Figure 2

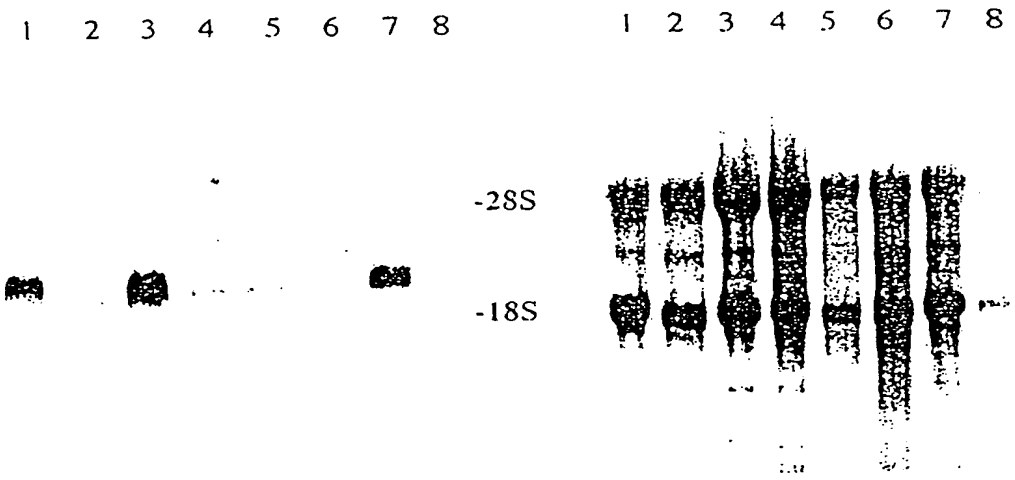


FIG 3A FIG 3B

A - nothern blot hybridisation with DIG-labled H19 probe (CDP-Star detection, exp. time-2min.).
B - RNA loading on the blot, methylen blue staining befor hybridisation.

# Effect of halofuginone on integrins

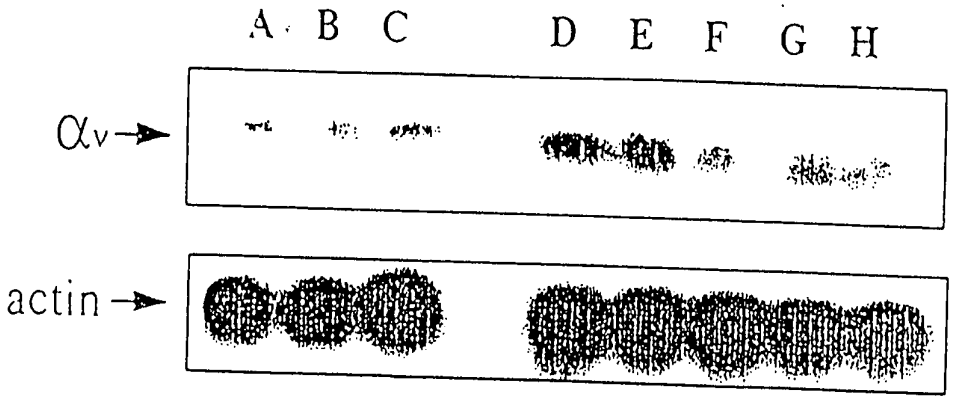


FIG 4

Effect of halofuginone on integrin gene expression

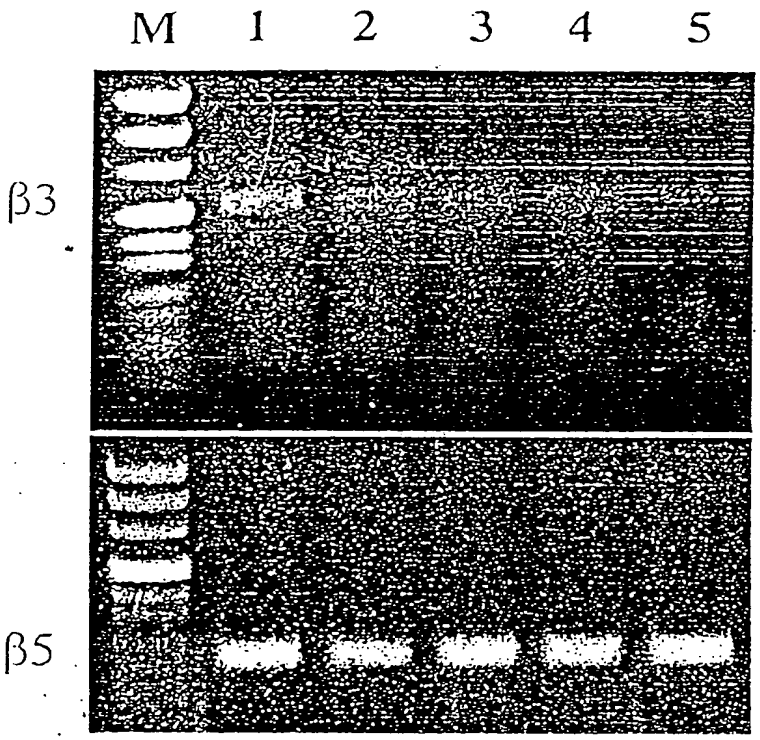


FIG 5

ARJ sheet 6 of 6



# Collagen Volume Quantitation in the Rat Heart

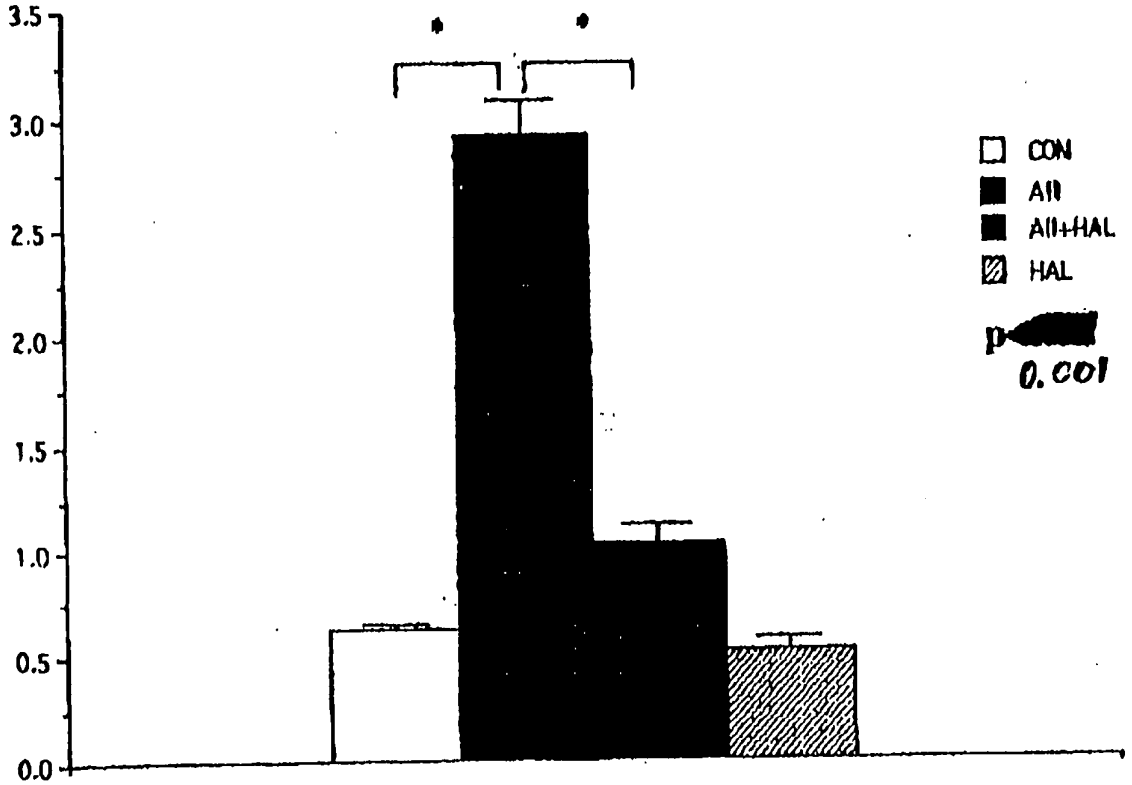


Figure 6